09/011167

PATENT

ATTORNEY DOCKET NO. RILE.001.00US

UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

RO Rec'd PCT/PTO 02FEB1998

In re application of: GEUZE *et al*

International Application No.: PCT/NL96/00317

International Filing Date: August 5, 1996

Priority Claimed: August 3, 1995

For: **CELL DERIVED ANTIGEN PRESENTING VESICLES**

Examiner: Not yet assigned

Art Unit: Not yet assigned

TRANSMITTAL FOR NEW PATENT APPLICATION UNDER 35 U.S.C. §371

BOX PCT
Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

CERTIFICATE OF EXPRESS MAILING

"Express Mail" Label No.: EM516292526US

Date of Deposit: 02-02-98

I hereby certify under 37 C.F.R. 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above and is addressed to BOX PCT, Commissioner of Patents and Trademarks, Washington, D.C., 20231.

Signature: Lynda Cunningham

Printed Name: LYNDA CUNNINGHAM

3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))

   a) [ ] is transmitted herewith (required only if not transmitted by the International Bureau).

   b) [X] has been transmitted by the International Bureau.

   c) [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).

6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))

   a) [ ] are transmitted herewith (required only if not transmitted by the International Bureau).

   b) [ ] have been transmitted by the International Bureau.

   c) [ ] have not been made; however, the time limit for making such amendments has NOT expired.

   d) [X] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. [X] An oath or declaration of the inventor(s) 35 U.S.C. 371(c)(4)) (unexecuted).

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CGT 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment.

[ ] A SECOND or SUBSEQUENT preliminary amendment.

14. [ ] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [X] Other items or information.

[X] A copy of the application as published along with a copy of the Amended Claims as transmitted with the International Preliminary Examination Report dated 11/19/97 are enclosed.

[ ] This application is a CIP of

17. [X] The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

[ ] USPTO was IPEA

[ ] All claims presented satisfied provisions of PCT Article 33(2) to (4) — $ 49.00/98.00

[ ] All claims presented did not satisfy provisions PCT Article 33(2) to (4) — $360.00/720.00

[ ] USPTO was ISA but not IPEA — $395.00/790.00

[X] USPTO was neither ISA nor IPEA

[ ] Search report has not been prepared by the European Patent Office or the Japanese Patent Office — $535.00/1070.00

[X] Search report has been prepared by the European Patent Office or the Japanese Patent Office — $465.00/930.00

Basic Fee Amount = $ 930.00

[ ] Surcharge of $130.00 for furnishing the oath or declaration later than

[ ] 20 months

[ ] 30 months

from the earliest claimed priority date (37 CFR 1.492(3)).

FOR:	Claims Filed	Extra Claims	Small Entity Rate	Fee	Other than a Small Entity Rate	Fee	Total Claim Fee
Total Claims	9	0	11.00		22.00	$	$ -0-
Independent Claims	4	1	41.00		82.00	$	$ 82
Multiple Dependent Claims Presented			135.00		270.00	$	$
TOTAL							$ -0-

Total Claim Fee = $82.00

[ ] Verified Small Entity Statement enclosed with filing.

[ ] Processing fee of $130.00 for furnishing the English translation later than

[ ] 20 months

[ ] 30 months

from the earliest claimed priority date (37 CFR 1.492(f)).

[ ] Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property.

Total Fees = $1012.00

[X] A check in the amount of **$1012.00** to cover the above fees is enclosed.

[ ] Please charge my Deposit Account No. 18-0020 in the amount of $ to cover the above fees.

A **duplicate** copy of this sheet is enclosed.

[X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-0020.

Respectfully submitted,

Date: January 30, 1998

By: Barbara Rae-Venter

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PATENT

ATTORNEY DOCKET NO. RILE.001.00US

UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

In re application of: GEUZE *et al*	)	Examiner: Not yet assigned
International Application No.: PCT/NL96/00317	)	Art Unit: Not yet assigned
International Filing Date: August 5, 1996	)	**PRELIMINARY AMENDMENT**
Priority Claimed: August 3, 1995	)	
For: **CELL DERIVED ANTIGEN PRESENTING VESICLES**	)	

BOX PCT
Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Applicants are submitting herewith a Preliminary Amendment in the above-referenced patent application entering National Stage from the PCT. Prior to examination of the application, the Examiner is respectfully requested to enter the following amendments.

CERTIFICATE OF EXPRESS MAILING

"Express Mail" Label No.: EM 516292526 US

Date of Deposit: 02-02-98

I hereby certify under 37 C.F.R. 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above and is addressed to BOX PCT, Commissioner for Patents and Trademarks, Washington, D.C., 20231.

Signature: Lynda Cunningham

Printed Name: LYNDA CUNNINGHAM

AMENDMENTS

In the Specification:

At page 1, line 22, change "1-chain" to –I-chain--.

At page 2, line 28, before "DS" insert --S--.

At page 7, line 2, change "motiv" to --motif--.

At page 10, line 27, change "duplo" to --duplicate--.

In the Claims:

Cancel Claim 1.

2. (Amended) The antigen presenting [Vesicle] vesicle according to claim [1] 13, wherein [comprising at least a biologically active part of an] said major histocompatibility [comlex] complex protein is derived from MHC class I or class II [or a derivative thereof].

3. (Amended) The [Vesicle] antigen presenting vesicle according to claim [2] 13 [which additionally comprises] further comprising at least [partly] partially processed antigens.

4. (Amended) The [Vesicle] vesicle according to claim 3 wherein said at least partially processed antigens [is] are presented in the context of [major histocompatibility complex] MHC class [1] I or class [2] II proteins.

Cancel Claim 5 without prejudice to renewal.

6. (Amended) The [Vesicle] antigen presenting vesicle according to [anyone of the aforegoing claims] claim 13, wherein said antigen presenting cell [which] is derived from a B-lymphocyte, a Langerhans cell, a macrophage or a dendritic cell.

Cancel Claims 7-8 without prejudice to renewal.

9. (Amended) A [Method] method for [the preparation of a] obtaining antigen presenting [vesicle according to anyone of claims 1-4,] vesicles having a membrane and a

major histocompatibility complex (MHC) protein, said method comprising the [steps] step of:

recovering a membrane-enriched fraction obtained by differential centrifugation of [membrane] membrane-containing fractions of cell culture supernatants or lysates of antigen presenting cells whereby [and recovery of the fraction] fractions containing said antigen presenting vesicles are obtained.

10. (Amended) A [Method] method for stimulating a T cell [response] comprising the step of contacting said T cell[s] with [a] the antigen presenting vesicle according to claim [3 or 4] 13.

Add the following new claims:

--11. A method for obtaining antigen presenting vesicles having a membrane and a major histocompatibility complex (MHC) protein derived from MHC class II, said method comprising the step of:

recovering a 70,000 x g pellet obtained by differential centrifugation of membrane-containing fractions of cell culture media or lysates of antigen presenting cells containing MHC class II, whereby fractions containing said antigen presenting vesicles are obtained.

12. A method for obtaining purified antigen presenting vesicles having a membrane and a major histocompatibility complex (MHC) protein derived from MHC class II, said method comprising the step of:

recovering a fraction having a buoyant density of 1.10 to 1.22 g/ml from a 70,000 x g pellet obtained by differential centrifugation of membrane-containing fractions of cell culture supernatants or lysates of antigen presenting cells containing MHC class II, whereby purified antigen presenting vesicles are obtained.

13. An antigen presenting vesicle free from its natural surroundings obtainable from an antigen presenting cell, comprising:

a membrane and a major histocompatibility complex (MHC) protein or a functional derivative or fragment thereof.--.

REMARKS

The amendments to the specification are to correct typographical errors. The remaining amendments are to put the claims from the PCT application into traditional US format and to correct typographical errors. Support for the amendments is in the claims as filed. Support for new Claims 11, 12 and 13 is found for example at page 9, lines 11-20; and page 2, lines 14 to page 4, line 3.

No new matter is added by the above amendments and the Examiner is requested to enter the amendments.

Respectfully submitted,

Date: February 2, 1998

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BRV/amp RILE.001.00US PRELIM AMEND 012998

PATENT ATTORNEY DOCKET NO.RILE.001.00US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Geuze *et al*	Examiner: Not yet assigned
Serial No.: 09/011,167	Art Unit: Not yet assigned
Filed: February 2, 1998	
For: **CELL DERIVED ANTIGEN PRESENTING VESICLES**	**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 C.F.R. §§ 1.9(f) & 1.27(d) -- NONPROFIT ORGANIZATION**

Assistant Commissioner for Patents
Washington, D.C. 20231

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

RIJKSUNIVERSITEIT te LEIDEN

Type of Nonprofit Organization:

[X] University or Other Institution of Higher Education

[ ] Tax Exempt Under Internal Revenue Service Code [26 USC 501(a) and 501(c)(3)]

[ ] Nonprofit Scientific or Educational Under Statute of State of the United States of America

(Name of State: _____)
(Citation of Statute: _____)

[ ] Would qualify as tax exempt under Internal Revenue Service Code [26 USC 501(a) and 501(c)(3)] if located in the United States of America

[ ] Would qualify as nonprofit scientific or educational under Statute of State of the United States of America if located in United States of America

(Name of State: _____)
(Citation of Statute: _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. § 1.9(e) for purposes of paying reduced fees under Section 41(a) or (b) of Title 35, United States Code with regard to the invention identified above and described in

[ ] the specification filed herewith

[X] application filed February 2, 1998, identified as Attorney Docket No.RILE.001.00US

[ ] patent no. _____, issued _____.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization regarding the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

* NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27)

Name: Universiteit Utrecht
Address: Universiteitsweg 100
3584 CG Utrecht
The Netherlands

[ ] Individual [ ] Small Business Concern [X] Nonprofit Organization

Name:
Address:

[ ] Individual [ ] Small Business Concern [ ] Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing S.J. Beuze

Title of Person Other than Owner Managing Director

Address of Person Signing Stationsweg 46, 2312 AV Leiden, The Netherlands

Signature

Date 27/08/1998

Title: Cell derived antigen presenting vesicles.

The invention relates to the field of immunology, especially the cellular responses of the immune system, more in particular to the induction of said responses by peptides presented in the context of major histocompatibility complexes I and/or II.

It is known that antigen presenting cells take up antigens through endocytosis, whereafter these antigens are cleaved into peptides which are presented at the surface of said antigen presenting cells in the context of a major histocompatibility complex. By this presentation on the surface the peptides derived from the original antigen can be recognized by for instance helper T-lymphocytes, further activating the cellular immune response.

Thus Helper T-lymphocytes recognize exogenous antigens bound to major histocompatibility complex (MHC) class II molecules expressed by a variety of antigen presenting cells (APCs) such as B-lymphocytes, macrophages and dendritic cells (1). Compelling evidence indicates that newly synthesized α and β subunits of MHC class II in association with the invariant chain (I-chain) are transported to intracellular compartments before reaching the plasma membrane (2,3). In these compartments the I-chain is degraded and MHC class II are potentially free to bind antigenic peptides arising from the degradation of antigens internalized by the APC (1, 4). We and others have shown that most of the intracellular MHC class II molecules reside in a lysosome-like, MHC-class II-enriched compartment (MIIC) which contains characteristic membrane vesicles and concentrically arranged membrane sheets (5, 6, 7, 8, 9, 10) . MIICs and the related CIIVs (11), likely represent the meeting point between MHC class II and antigenic peptides (8,12). Once loaded with peptide, MHC class II molecules are transferred to the cell surface via an unknown pathway for presentation to T-lymphocytes.

Electron microscopy of immunogold labeled ultra thin cryosections from several human B-lymphoblastoid cell lines revealed MIICs whose surrounding membrane was contiguous with the plasma membrane in an exocytotic fashion and showed extracellular vesicles reminiscent of those present in non-fused MIICs (Figure 1A and B). Similar secretion of vesicles, termed exosomes, has been described for reticulocytes (13). Exosomes from B cells immunolabeled for the lysosomal membrane proteins LAMP1 (Figure 1 B) and CD63 (not shown) known to be expressed in MIICs (5, 6). Both LAMP1 and CD63 were absent from the rest of the plasma membrane. Scarce labeling for MHC class II was associated with the limiting membrane of the fused MIICs but MHC class II was enriched in the externalized exosomes (Figure 1A and B). To test the release of MIIC contents further, B cells were allowed to internalize 5 nm gold particles conjugated to Bovine Serum Albumin (BSAG), and were then washed and reincubated in the absence of BSAG. Exosomes associated with previously endocytosed BSAG began to appear in exocytotic profiles after 30 min of uptake (10 min pulse and 20 min chase) (Figure 1B) and were abundant after 50 min (10 min pulse and 40 min chase) (Figure 1A). We conclude that multivesicular MIICs of human B-cell lines can fuse with the plasma membrane thereby releasing MHC class II-rich exosomes into the extracellular milieu.

For a further characterization, exosomes were isolated from the culture media of the human B cell line RN by differential centrifugation (Figure 2). Pelleted membranes were analyzed by DS-PAGE and Western blotting. After removal of cells, the majority of MHC class II-containing membranes sediment at 70.000 g (Figure 2 A, lane 6). The 70.000 g pellets were composed of a homogeneous population of vesicles labeled for MHC class II (Figure 2 B). The vesicles were morphologically similar to those present in MIICs and in exocytotic profiles of sectioned cells (Figures 1 A and B): their size ranged from 60 to 80 nm. To obtain biochemical evidence that the secreted MHC class II is membrane bound, 70.000 g pellets were fractionated by floatation in linear

sucrose gradients (14). Western blot analysis of the non-boiled and non-reduced gradient fractions showed that MHC class II molecules floated to an equilibrium density of 1.13 g/ml, confirming their association with membrane vesicles (Fig. A). MHC class II molecules recovered from the gradient fractions were predominantly in the SDS-stable, compact form indicating their stabilization by bound peptides (15). Together, these results show that the secreted MHC class II is associated with membrane vesicles and has bound peptides. To determine the kinetics and the extent to which newly synthesized MHC class II molecules are released into the medium, RN cells were metabolically pulse-labeled for 45 min. with [^{35}S]-methionine and chased for up to 24 hours in the absence of label (16). After pulse-labeling MHC class II was immunoprecipitated as SDS-unstable α-β-1-chains complexes (Fig.3 B, lane 0). At 6 hours of chase part of MHC class II molecules were converted to SDS-stable, α-β-peptide complexes consistent with the kinetics reported for other human B cell lines (2, 17). Recovery of [^{35}S]-compact MHC class II from pelleted exosomes started at 12 hours and amounted 10 + 4% (n=5) of the total newly synthesized MHC class II after 24 hours of chase. The relatively slow rate by which newly synthesized MHC class II was secreted into the medium suggests that insertion from the limiting membrane of MIICs into the plasma membrane during exocytosis is probably not the only pathway by which MHC class II molecules are delivered to the cell surface. To test the possibility that the vesicles recovered from the medium represented shed plasma membrane fragments or cell debris instead of exosomes, cells and exosome preparations were biotinilated and the patterns of the biotinilated proteins were studied by Western blotting with ^{125}I-Streptavidin (18). Figure 3 C reveals differential patterns of biotinilated proteins in exosomes and plasma membranes. Whereas plasma membranes show a broad spectrum of biotinilated proteins (Figure 3C, lane 2), two proteins are enriched in exosomes (Figure 3C, lanes 3 and 4). Immunoprecipitation of the biotinilated exosomal proteins with

a monoclonal anti-class II antibody (19) identified these proteins as MHC class II (α and β subunits (Figure 3C, lane 1). Furthermore, the exosomes contain two minor bands at higher molecular weight which are not clearly detected in plasma membranes (Figure 3C, lanes 3 and 4). These proteins were also immunoprecipitated with the anti-class II antibody (Figure 3C, lane 1). To test the unlikely possibility that plasma membrane fragments eventually present in the 70.000 g pellets contributed to the enrichment of MHC class II in exosomes, biotinilated cells were homogenized and the homogenates were processed as the cell culture supernatants (18). Very low amount of membranes are pelleted at 70.000 g and these show a pattern of biotinilated proteins matching that of total plasma membrane, as expected (Figure 3C, lane 5). When the cells were metabolically labeled with [35S]-methionine for 45 min. and chased for up to 24 hours (16), the [^{35}S]-Transferrin receptor (TfR) ([^{35}S]-TfR) did not appear in exosomes at any chase time (data not shown). TfR is present at the plasma membrane of B cells but is absent from MIIC (8, 10). Together, these observations emphasize that exosomes are not derived from shed plasma membranes but represent an unique population of MHC class II- enriched membrane vesicles.

Since the luminal domain of MHC class II molecules is exposed at the outside of exosomes (20), exosomes may be able to present antigens to T cells. To test this hypothesis, isolated exosomes were allowed to bind peptide 418-427 from the model antigen HSP 65 of Mycobacterium Leprae. The exosome preparations were then added to the T cell clone 2F10 which recognizes this peptide in the context of HLADR15 (21). In a parallel experiment, RN cells were allowed to endocytose HSP65 protein continuously for 24 hrs, washed, and incubated in the absence of antigen for another 24 hrs (22). Both, exosomes incubated with antigenic peptide (Figures 4 A and C) and exosomes derived from cells that were pre-incubated with antigen (Figures 4 B and D) were able to induce a specific T cell response (23). A half maximal response was obtained with an amount of exosomes secreted by 3 x 10^5 RN cells in 24 hours

(Fig.4 D). In comparison $2x10^4$ intact RN cells were necessary to achieve the half maximal response (Fig.4 B, 24). The responses observed were DR restricted. Anti-HLA-DR antibody blocked T cell proliferation completely, whereas antiHLA-DP was ineffective (Figs 4 B and D). From these data we conclude that culture media of B cells provide for a source of MIIC-derived microvesicles (exosomes) that can induce T cell responses by themselves (25).

Exocytosis of MIIC vesicles by B-lymphocytes is reminiscent of the exocytosis of the vesicles contained in the cytolytic granules of cytotoxic T-lymphocytes (CTLs) (26). Both MIICs and cytolytic granules have lysosomal characteristics and contain internal membranes. The internal vesicles of cytolytic granules are exocytosed by the CTLs upon CTL-target cell interaction and presumably have a role in the killing of target cells (26). Whether B-cell exosomes also have an extracellular role *in vivo* remains to be established. It has been suggested that follicular dendritic cells acquire MHC class II molecules released from surrounding B cells by an unknown mechanism (27). It is worth studying the possibility that exosomes serve as carriers of MHC class II-peptide complexes between different cells of the immune system. Whether physiological APCs like dendritic cells and macrophages generate exosomes has to be studied (28). However, secretion of lysosomal contents by macrophages has been documented and macrophage tubular lysosomes are rich in MHC class II and contain membrane vesicles (29). It can be speculated that *in vivo*, exosomes may function as transport vehicles for MHC class II-peptide complexes responsible for maintenance of long term T cell memory or T cell tolerance. Finally, since exosomes can easily be obtained and are capable of presenting antigens specifically and efficiently, it is worth exploring their usefulness as biological vehicles in immunotherapy.

The invention therefore provides an antigen presenting vesicle free from its natural surroundings obtainable from

antigen presenting cells, such as B-cells, macrophages or dendritic cells, especially Langerhans cells of the epidermis.

These vesicles preferably will contain major histocompatiblility complex (MHC) I and/or II, most preferably loaded with a peptide derived from or corresponding to an antigen which can be processed by antigen presenting cells.

It has been tried before to produce similar vesicles synthetically, for instance in the form of liposomes, but these attempts have sofar not been successful. Now that we have surprisingly found that there are counterparts of said liposomes in nature, these counterparts can of course be used in any intended application of said liposomes.

The major advantage of the vesicles according to the invention is of course that they will automatically comprise all the necessary elements for antigen presentation. Further analysis of the vesicles, once discovered will therefore result in a better understanding of which elements are essential for said presentation on said vesicles. It will then of course be possible to arrive at vesicles according to the invention in other ways then by isolation from cells. The invention therefor does encompass all antigen presenting vesicles which comprise the essential elements for presenting such antigens, regardless of the way they are produced or obtained.

One may for instance think of synthetically prepared liposomes, provided with at least biologically active parts of (recombinant) MHC I or II, optionally provided with processing agents for antigens to be presented in the context of said MHC. Of course cells which produce these vesicles can also be provided with recombinant MHC I or II encoding genes, so that the desired MHC's will be present on the eventually resulting vesicles, etc.

Although vesicles which present peptides in the context of MHC I or II are preferred, it is also very useful to produce vesicles which do have the MHC's on their surface, but without a peptide being present therein. These vesicles can

then be loaded with desired peptides having the right binding motiv to fit in the respective MHC.

The first and perhaps foremost use of these vesicles that comes to mind is of course mimicking their role in nature, which is the presentation of peptides as antigens, for the stimulation of for instance T-cells. Thus the vesicles according to the invention can be very suitably used in for instance vaccines. These vaccines can be designed to elicit an immune response against any proteinaceous substance which has peptide antigens that can be presented in the context of MHC.

The vaccines may of course comprise suitable adjuvants, if necessary, carriers, if necessary, ecxipients for administration, etc.

The vaccines can be used in the treatment or prophylaxis of many disorders, such as infections, immune disorders, malignancies, etc.

Very important applications will of course be the treatment or prophylaxis of AIDS, eliciting immuneresponses agains tumours and the like.

Another important application of the vesicles according to the invention is that they may be used to induce tolerance to certain antigens, for instance by giving large doses of the vesicles orally.

Based on the description of the invention and specifically referring to the following experimental part illustrating the invention the person skilled in the art will be able to find further uses of the vesicles according to the invention without departing from the spirit of the invention.

Legends to Figures:

Figure 1:

MIICs are exocytotic compartments. T2-DR3 cells were incubated in the presence of 5 nm BSAG for 10 min., washed, chased for 40 min. and processed for cryoultramicrotomy as described (30). Ultrathin cryosections were immunolabeled with a rabbit polyclonal anti-class II antibody (5) and antibody binding sites were visualized with protein A conjugated to gold (PAG with sizes in nm indicated on the figures). MHC class II labeling is present at the limiting membrane of the exocytotic profile and on the exosomes. The profile also contains abundant re-externalized BSAG particles. PM: plasma membrane. B, RN cells were pulsed with BSAG for 10 min. and chased for 20 min. Ultrathin cryosections were double-immunolabeled with anti-class II antibody and with a monoclonal anti-LAMP1 antibody (31) as indicated. One of two neighboring profiles is shown, exocytotic profile containing BSAG and numerous exosomes labeled for MHC class II and LAMP1.Bars, 0.1 μm.

Figure 2:

Isolation of exosomes from cell culture media. A, RN cells were washed by centrifugation and re-cultured in fresh medium for 2 days. Cell culture media (35 ml) containing 2-5 $x10^8$ RN cells were centrifuged twice for 10 min. at 300 g (lane 1, first run; lane 2, second run). Lane 1 contains material from 0.6 x 10^6 cells. Membranes in the culture medium from 2-5 x 10^8 cells were pelleted by sequential centrifugation steps: twice at 1200 g (lane 3 and 4), and once at 10.000 g (lane 5), 30.000 g (lane 6) and 100.000 g (lane 7). The pellets were solubilized at 100°C under reducing conditions and analyzed by Western blotting using [^{125}I]-protein A. Per lane, samples equivalent to 1 $x10^6$ cells were loaded. MHC class II α and β chains were recovered mainly from the cells (lane 1) and from the 70.000 g pellet (lane 6). B, whole mount electron microscopy of the 70.000 g pellet

immunogold labeled for MHC class II. The 70.000 g pellet was resuspended in RPMI medium, adsorbed to Formvar-carbon coated EM grids, fixed with 0.5 % glutaraldehyde in 0.1 M phosphate buffer, immunolabeled with rabbit polyclonal anti-class II antibody and 10 nm PAG and stained using the method described for ultra-thin cryosections (30). The pellet is composed of 60-80 nm vesicles showing abundant MHC class II labeling. Bar, 0.2 µm

Figure 3:

A, MHC class II present in the media are membrane bound. Membranes pelleted from culture media at 70.000g after differential ultracentrifugation were fractionated by floatation on sucrose gradients, and the non-boiled and non-reduced fractions analyzed by SDS-PAGE and Western blotting with the rabbit polyclonal anticlass II antibody (17). MHC class II molecules were recovered in fractions 5 to 12 corresponding to densities of 1.22-1.10 g/ml. The majority of MHC class II was in the SDS-stable compact form with a MW of ~ 56-60 kD (Coc/β).

B, Release of newly synthesized MHC class II molecules. RN cells were pulse-labeled with [^{35}S] methionine for 45 min. (lane 0) followed by chases in the absence of label for 6, 12 and 24 hours. MHC class II molecules were immunoprecipitated from lysates of the cells and pelleted exosomes with the monoclonal DA6.231 anti-class II antibody (18). Immunoprecipitated MHC class II molecules were dissociated from the sepharose beads at non-reducing conditions at room temperature and analyzed by SDS-PAGE and fluorography. After pulse-labeling (0), MHC class II immunoprecipitated from the cells as SDS-unstable complex of α-β-invariant chain. SDS-stable α-β dimers were recovered from the cells after 6 hours of chase and the signal increased thereafter. In the exosomes pellets SDS-stable αβ dimers started to appear at 12 hours. C, Exosomes and plasma membrane display different patterns of biotinilated proteins (18). In plasma membranes (lane 2) and experimentally produced remnants of plasma membranes (18) many

biotinilated proteins are detected with ^{125}IStreptavidin (lane 5). In exosomes (lanes 3 and 4, show increasing concentrations of exosomes, respectively) two major proteins with a MW of 60-70 kD are detected. Lane 1 shows the immunoprecipitation of biotinilated class II α and β chains from exosomes Iysates. In these assay the higher electrophoretical mobility of α and β chains is due to their efficient binding to biotin. Two minor bands at a MW of 200-300 kD are detected in exosomes (lanes 1, 3 and 4, arrows) and are absent from the plasma membrane.

Figure 4:

Presentation of HSP 65 antigen by HLA-DR15 positive RN B cells and exosomes to the CD4$^+$ T cell clone 2F10 (22). Proliferative responses to naive cells (A), to cells pre-incubated with antigen (B), to exosomes derived from naive cells (C) and to exosomes derived from cells pre-incubated with antigen (D). The closed symbols show proliferation measurements after addition of HSP 65 derived peptide (418-427), the open symbols where peptide was not added. HLA-class II restriction was determined by adding 10 µg/ml anti-DR antibody (triangles), anti-DP (circles), or no antibody (squares). The exosomes at the highest concentration were derived from media of 1.6 x 10^6 cells. All assays were performed in triplicate and results are expressed in cpm [^{3}H]-thymidine incorporated into T cells. The SEM for triplicate cpm measurements was less then 10%. Results shown form a representative example of experiments performed in duplo.

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14. The 70.000 g pellet obtained after differential centrifugation of the cell culture supernatants of RN B

lymphoblastoid cells was resuspended in 5 ml of 2.5 M sucrose, 20 mM Hepes/NaOH pH 7.2. A linear sucrose gradient (2 M-0.25 M sucrose, 20 mM Hepes- NaOH, pH 7.2) was layered over the exosome suspension in a SW27 tube (Beckman) and was centrifuged at 100.000 g for 15 hrs. Gradient fractions (18 x 2 ml) were collected from the bottom of the tube, diluted with 3 ml PBS and ultracentrifuged for 1 hr at 200.000 g using a SW50 rotor (Beckman). The pellets were solubilized at room temperature in SDS-sample buffer lacking -- mercaptoethanol and analyzed by SDS-PAGE and Western blotting using ^{125}I-Protein A.

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16. RN cells were pulsed for 45 min. with 50 Mbq/ml [35S]-methionine (Tran-Slabel, ICN, CA) and chased for different periods of time (5x107 cells per time point). After pulse-chase labeling, the cells were pelleted by centrifugation for 10 min. at 300 g. The supernatants were collected and centrifuged for 5 min. at 10.000 g and then for 30 min. at 200.000 g in a SW60 rotor (Beckman). Cells and the 200.000 g pellets were lysed and MHC class II and TfR were immunoprecipitated from equal samples of the lysates. TfR was immunoprecipitated as described previously [W. Stoorvogel, H. J. Geuze, J. M. Griffith, A. L. Schwartz, G. J. Strous, J. *CellBiol.* **108**, 2137-2148 (1989)]. MHC class II was quantitated using a Phosphoimager.

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18. RN cells (2 x 10^8) were washed 3 times with ice cold PBS and incubated for 30 min. at 0°C with 1mg/ml Sulfo-NHS-biotin (Pierce). Biotin was quenched for 30 min. with 50 mM NH4 Cl . After washing with ice cold PBS, half of the cells were solubilized in SDS-sample buffer supplemented with β-mercaptoethanol. The remaining biotinilated cells were homogenized. The homogenates were centrifuged and

ultracentrifuged identically to the cell culture supernatants and the 70.000 g pellets solubilized in SDS-sample buffer supplemented with β-mercaptoethanol (control for plasma membrane remnants). Exosome preparations (70.000 g pellets of cell culture media from 2 x 10 8 cells) were biotinilated as described above and solubilized in SDS-sample buffer supplemented with β-mercaptoethanol. MHC class II was immunoprecipitated from a sample of biotinilated exosomes with the monoclonal anti-class II antibody DA6.231 (19). The biotinilated cell membranes, biotinilated exosomes and immunoprecipitated MHC class II were analyzed by SDS-PAGE and Western blotting with ^{125}I-Streptavidin.

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20. The internal MIIC vesicles are formed by inward budding of the limiting membrane of MIICs (see figures 16 and 17 in reference [6] similar to the process described for multivesicular bodies in other cell types [B. van Deurs, P. K. Holm, L. Kayser, K. Sandvig, S. H. Hansen, *Eur. J. Cell Biol.* 61, 208-224 (1993)].

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22. The EBV-B cell lines RN (HLA-DR 15+) and JY (HLA-DR15-) were incubated in the presence or absence of purified HSP 65 protein from Mycobacterium Leprae (50µg/ml) [J.E.R. Thole, et al., *Microbial Pathogenesis* 4, 71-83 (1988)] for 4 hr in 10 ml serum free RPMI at 2 x 10^6 cells /ml, followed by the addition of 30 ml RPMI supplemented with 10% fetal calf serum (FCS) for 20 hr at 37°C. The cells were then washed to remove free antigen and incubated further for 24 hrs in RPMI/10% FCS medium at 37°C. Exosomes were prepared by differential centrifugation (Figure 2) and the efficiency of HSP 65 antigen presentation was measured by culturing 10.000 cells of

the T cell clone 2F10 with irradiated (6.000 rad) EBV cells. B cells or exosomes resuspended in 100 µl IMDM /10% pooled human serum were added to the T cell clone (50 µl IMDM /10% pooled human serum per well) in 96 well flatbottom microtitre plates (Costar, The Netherlands) for 4 days at 37°C, 5% C02 in humidified air. When indicated, 5 µg/ml of HLA-DR15 restricted epitope of HSP65 (peptide 418-427) was added to the exosomes. Sixteen hours before termination 0.5 µCi of [^{3}H]-thymidine was added to the wells. The cells were then harvested on glass fiber filters using an automatic cell harvester and the [^{3}H]-thymidine incorporation into cell DNA was determined by liquid scintillation counting. The results are expressed as the mean of triplicate measurements).

23. As a control, exosomes were prepared from culture media of an equivalent amount of DR15-negative JY cells that have been incubated or not with antigen. JY cells secreted an equivalent amount of exosomes but these were ineffective in stimulating T cell proliferation.

24. From these data exosomes appear to be 16 times less efficient in antigen presentation. However, in antigen presentation assays contact between B and T cells may be more efficient due to sedimentation of cells.

25. Exosomes isolated from the culture medium of the murine B cell line TA3 (1-E^{k+}) incubated in the presence a RNase-derived peptide (aa 90-105) were also capable of stimulating IL2 secretion by WA.23 cells.

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P. J. Peters, et al., J. *Exp. Med.* **173**, 1099-1109 (1991 b).

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28. A number of studies documented the presence of intact MHC class II molecules in 100.000 g fractions from B cell culture media and their association of with membrane

lipids [S. G. Emerson, R. E. Cone, J. *Immunol.* **122**, 892-899 (1979);
D. H. Sachs, P. Kiszkiss, K. J. Kim, J. *Immunol.* **124**, 2130-2136 (1980); S. G. Emerson, R. E. Cone, J. *Immunol.* **127**, 482-486 (1981)]. Our present observations shed new light on these data and suggest that the released MHC class II molecules were likely derived from secreted exosomes.

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Ren/PCT 0493

Amended claims

1. Antigen presenting vesicle free from its natural surroundings obtainable from the supernatant of a culture of antigen presenting cells.

2. Vesicle according to claim 1, comprising at least a biologically active part of an major histocompatiblity comlex class I or class II or a derivative thereof.

3. Vesicle according to claim 2 which additionally comprises at least partly processed antigens.

4. Vesicle according to claim 3 wherein processed antigen is present in the context of major histocompatibility complex 1 or 2.

5. Vesicle according to anyone of the aforegoing claims for use as a therapeutical.

6. Vesicle according to anyone of the aforegoing claims which is derived from a B-lymphocyte, a macrophage or a dendritic cell.

7. Vaccine composition comprising a vesicle according to anyone of claims 1-4 together with a usual adjuvans or carrier.

8. Use of a vesicle according to anyone of claims 1-4 in the preparation of a medicament for the treatment or prophylaxis of immune disorders or infections.

9. Method for the preparation of a vesicle according to anyone of claims 1-4, comprising the steps of differential centrifugation of membrane fractions of cell culture supernatants or lysates and recovery of the fraction containing said vesicles.

10. Method for stimulating a T cell response comprising the step of contacting T cells with a vesicle according to claim 3 or 4.

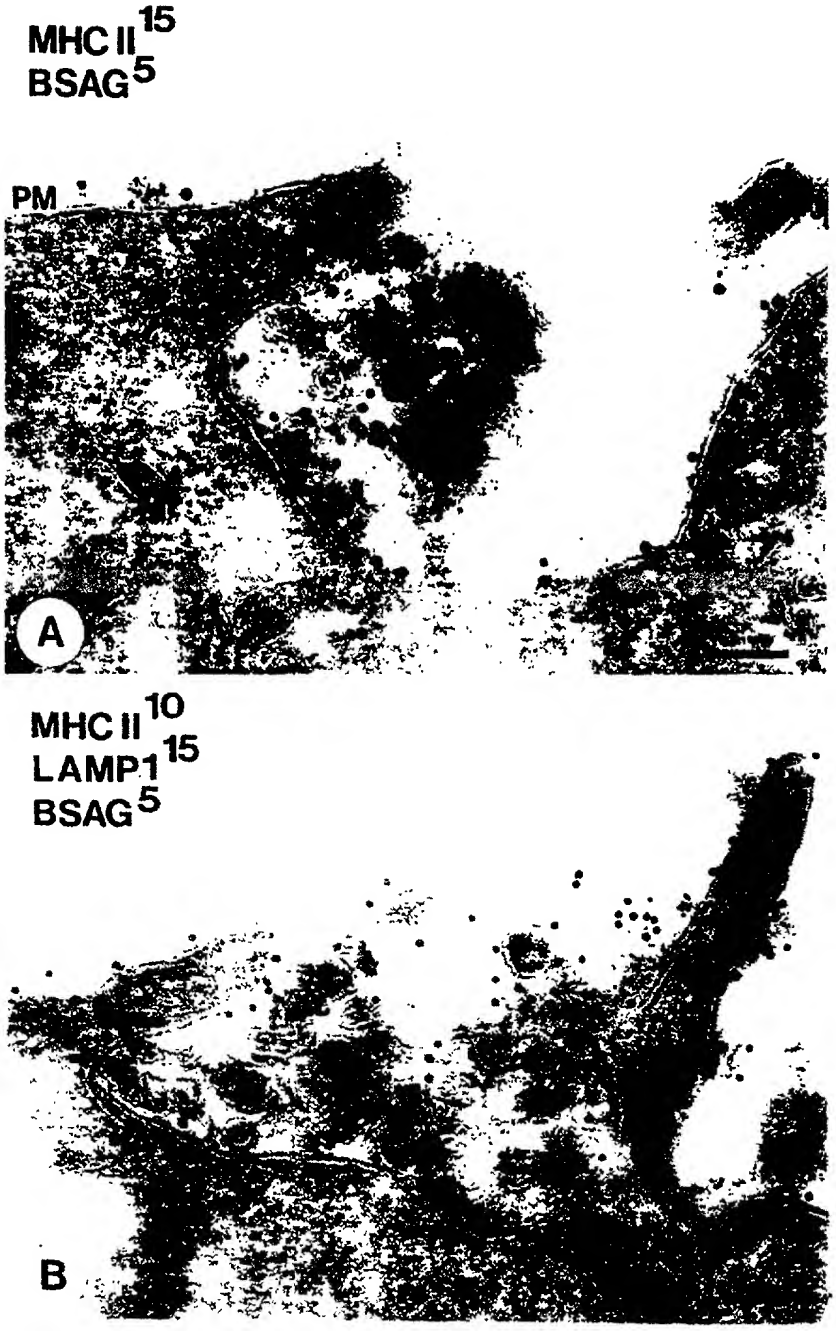

FIG. 1

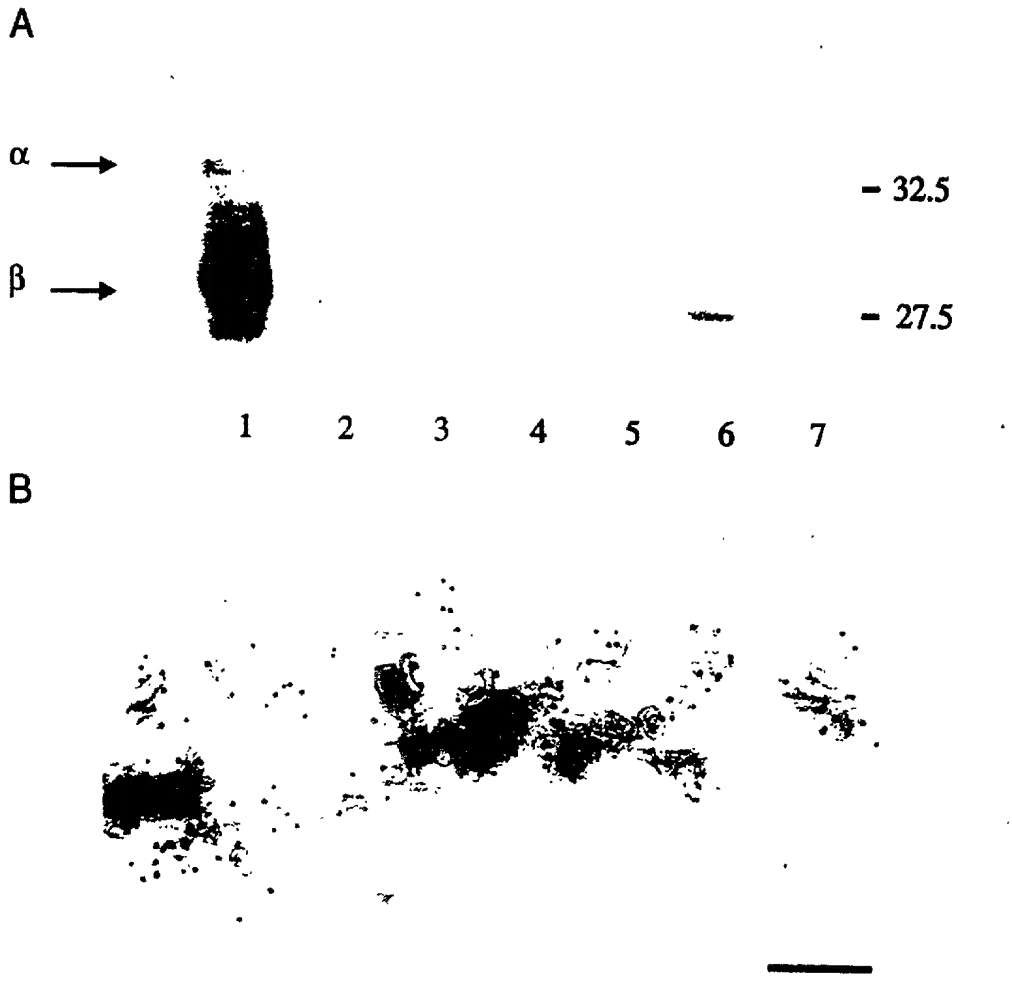

FIG. 2

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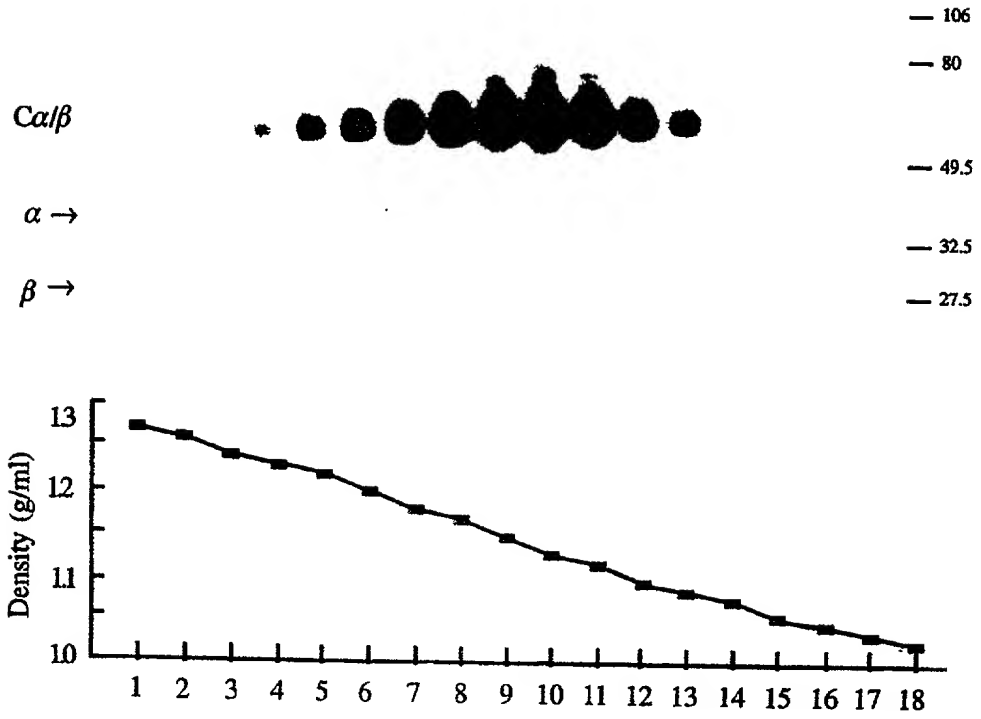
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β →
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80
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27.5
Density (g/ml)
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12
11
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

FIG. 3

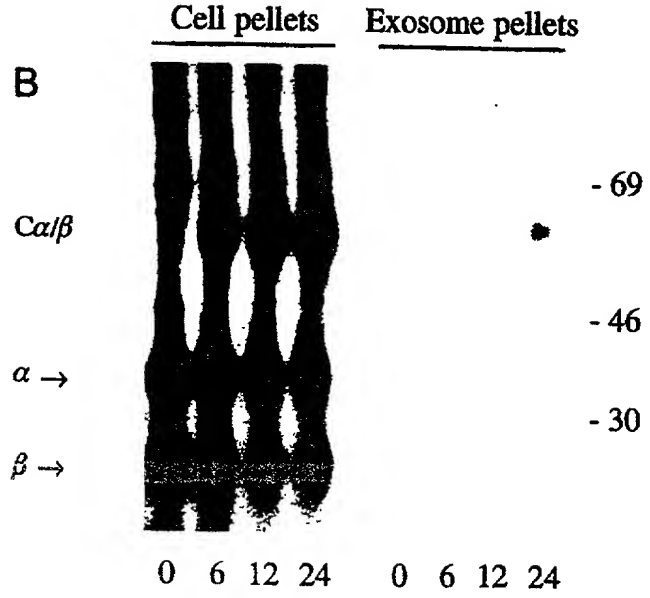


FIG. 3

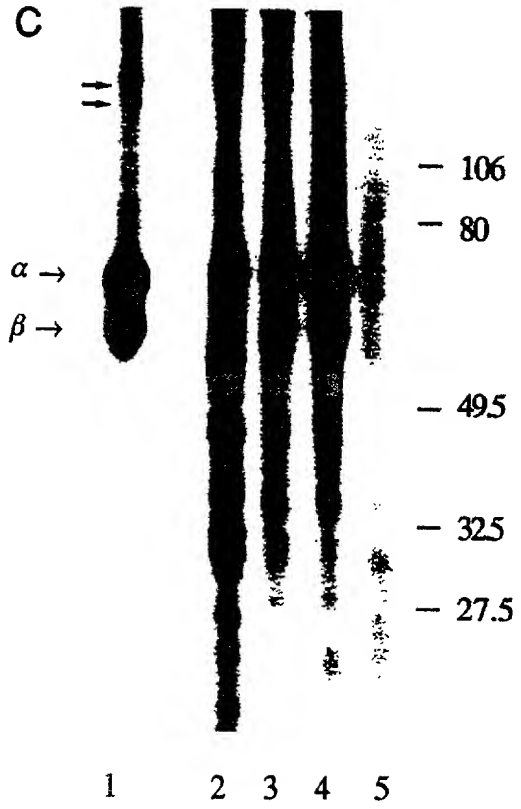
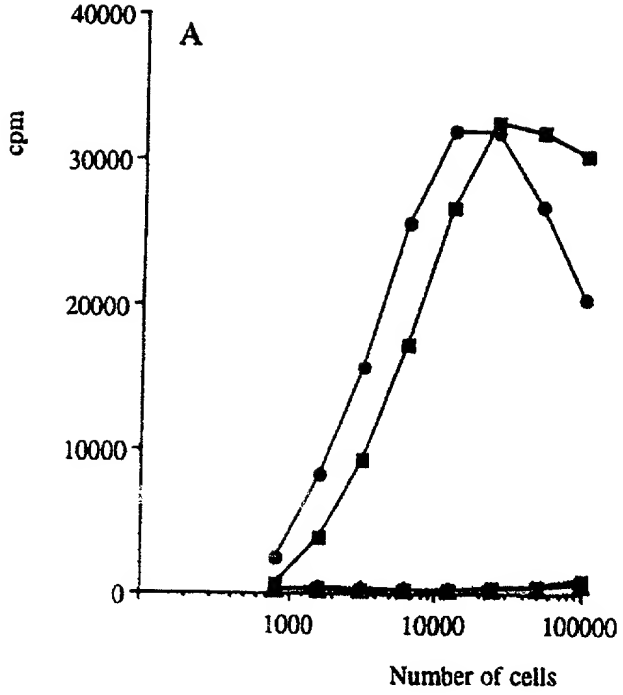


FIG. 3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Number of cells

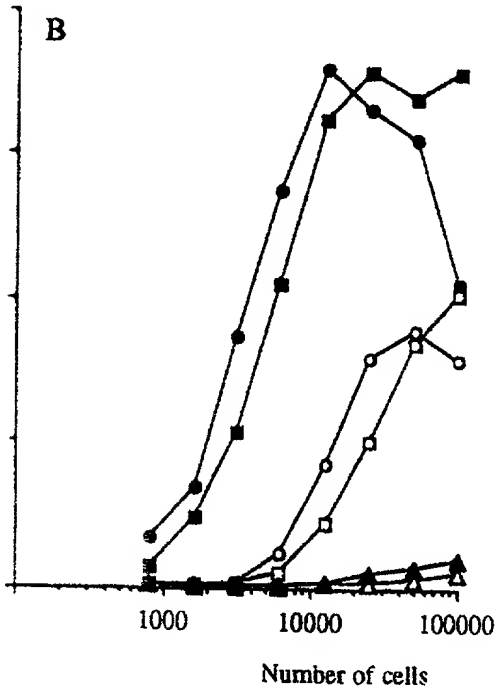
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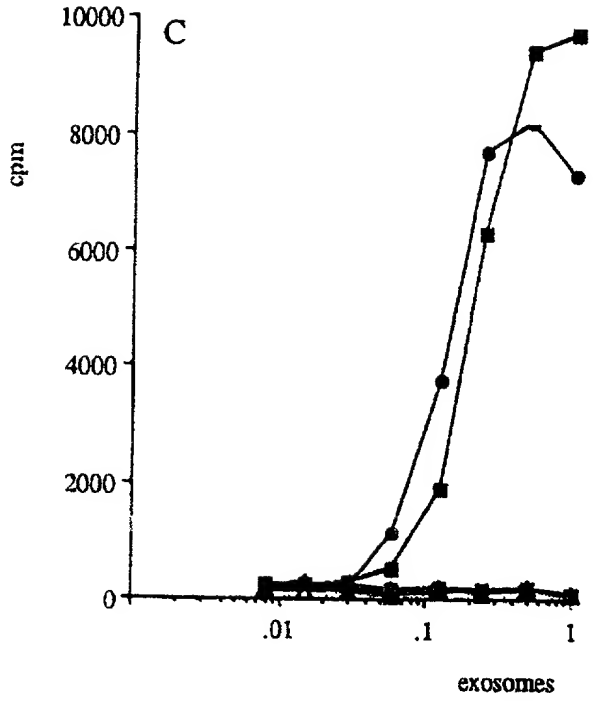
FIG. 4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1
exosomes

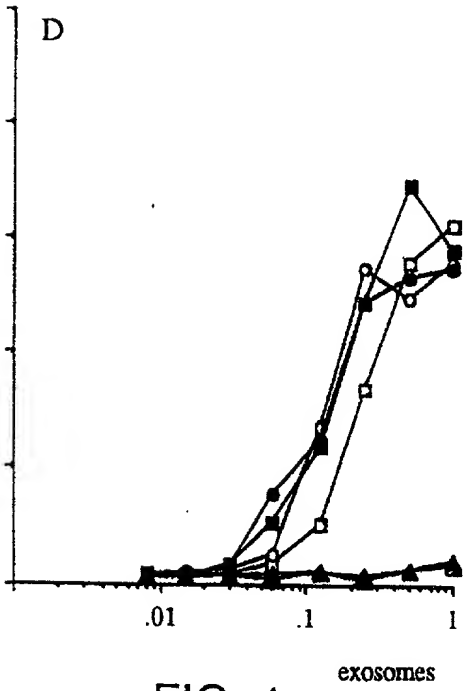
D
.01
.1
1
exosomes

FIG. 4

PATENT

ATTORNEY DOCKET NO. RILE.001.00US

COMBINED INVENTOR
DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

CELL DERIVED ANTIGEN PRESENTING VESICLES

the specification of which

(check one)
- [ ] Is attached hereto.
- [ ] Was filed on _____ and has been assigned Serial Number
- [X] Was filed on February 2, 1998, as Attorney Docket No. RILE.001.00US.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known to me to be material to patentability of this application as defined in Title 37, Code of Federal Regulations, §1.56 and, if applicable, all such information under 37 CFR § 1.56 which became available between the national or PCT International filing date of the prior application and the filing date of this application.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
95202123.6	EP	03/08/1995	[X]	[ ]
(number)	(Country)	(Day/Month/Year Filed)	Yes	No
Prior Foreign Application(s)			Priority Claimed	
(number)	(Country)	(Day/Month/Year Filed)	[ ] Yes	[ ] No
Prior Foreign Application(s)			Priority Claimed	
(number)	(Country)	(Day/Month/Year Filed)	[ ] Yes	[ ] No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC §112 I acknowledge the duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56, which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status)
* PCT/NL96/00317	5 August 1996	Published

* designating the U.S.

I hereby appoint:

4

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as my attorneys or agents with full power of substitution and revocation to prosecute my above-identified application for Letters Patent and to transact all business in the Patent Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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RILE.001.00US DECPOA 012998

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
[37 CFR 1.9(f) and 1.27(d)] -- NONPROFIT ORGANIZATION

Applicant or Patentee: C.J.M. MELIEF & J.J. GEUZE Docket No.
Serial or Patent No.:
Filed or Issued: 2 February 1998
For: Cell derived antigen presenting vesicles

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: Universiteit Utrecht
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TYPE OF ORGANIZATION

[X] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC 501(a) and 501(c) (3)]
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OR THE UNITED STATES OF AMERICA
(NAME OF STATE)
(CITATION OF STATUTE)
[ ] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC 501(a) and 501(c) (3)] IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE)
(CITATION OF STATUTE)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) or (b) of Title 35, United States Code with regard to the invention entitled Cell derived antigen presenting vesicles

by inventor(s) C.J.M. MELIEF & J.J. GEUZE

described in

[ ] the specification filed herewith
[X] application serial no. , filed 2 February 1998
[ ] Patent no. , issued

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27).

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ADDRESS Stationsweg 46, 2312 AV Leiden, the Netherlands
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [XX] NONPROFIT ORGANIZATION

NAME
ADDRESS
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC §1001, and may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Drs. J.L.A.M. Halkes
TITLE IN ORGANIZATION Managing Director
ADDRESS OF PERSON SIGNING Faculty of Medicine, University Utrecht, P.O. Box 80.030, 3508 TA Utrecht, The Netherlands

SIGNATURE DATE 19 01 1998